GENERAL SOP

A. Objective

1. To determine the prevalence of abnormalities in frog populations on National Wildlife Refuges.

B. Procedures

1. Scout for suitable amphibian breeding areas on refuge. The timing will be region specific, but generally begins in mid-late April.

2. Evaluate sites using the **Site Assessment SOP**.

3. For each suitable site selected, fill out a **Site Characterization Form**:

   - Assign a unique 5 character Site ID to all sites that will be monitored throughout season using the 3 character **Refuge ID Codes**, followed by sequential two digit numbers (ex: FSL01) [Note: the Refuge Code has been changed from a 2 character code to a 3 character code to be consistent with other FWS databases].

   - Take digital photographs of the site that best represents the habitat.
     - Name photos using standard format: FSL01-N-050103

   (Site ID – cardinal direction facing when photo was taken – date photographed without dashes)
     - Take additional photos throughout season to document any significant changes.
       If digital camera is not available, submit to your regional coordinator:
       (in order of preference): prints, 35 mm slides, or film negatives

   - Collect position data using a GPS unit and record on **Site Characterization Form**.
     - Required datum & format: WGS 84 in decimal degrees (hddd.ddddd°)
     - Required metadata: Make/model of GPS unit used.

4. Monitor selected sites for developing amphibians regularly once tadpoles are found. Record the following data on standard **Data Collection Forms** on each and every site visit:
   - Refuge Name, Site ID, Date, Collectors, Start & End time, ambient air and water temp (C°)
   - Note the presence, number and average water depth for any anuran egg masses (ID egg masses to species when possible).
   - If tadpoles are present, determine species & Gosner stage on 10 tadpoles for all species found.
   - Record any incidental species encountered (particularly potential predator species).
   - Record comments regarding when and why (or why not) a site should be revisited.

5. The goal is four full metamorph collections/refuge/season. Depending on the species of frogs in your region, you may or may not attain this goal. If possible, you should make two collections of between 50-100 metamorphs/cohort from a minimum of two sites per refuge each season. Collections can consist preferably of two distinct cohorts of the same species from each site or of two different species through the season from each site. Given the highly unpredictable nature of the weather and frog breeding activity, to ensure the minimum collections are made, it is advisable to aim at collecting multiple cohorts from multiple species from as many sites as possible. Select enough sites to monitor throughout the season to increase your chances of success.

6. Sites must fall within the refuge boundaries and whenever possible should be selected to reflect areas on the refuge with suspected contaminant inputs as well as those without such inputs. Clearly, you will have to collect "where the frogs are" which may or may not coincide with our selection criteria. Metamorphs should be collected using the **Capture SOP** and processed using the **Data Collection SOP.** All data should be recorded using standard **Data Collection Forms**.

7. If abnormal metamorphs are found, they should be documented with digital photographs and an **Abnormal Frog Form** for each individual. Consult your regional coordinator to determine if they need to be sent for diagnostics. If specimens are targeted for diagnostics, then prepare specimens using either the **Specimen Preservation SOP** or ship live according to the appropriate **Shipping SOP**. Track all abnormal specimens carefully on **Specimen Log** and copy your regional coordinator on all documentation that accompanies shipments.

8. Communicate any difficulties you encounter throughout the season to your regional coordinator as soon as possible. A regional teleconference with all project leaders may be scheduled to discuss sampling progress, diagnostic needs for abnormal specimens and any problems the group may have encountered.

9. Reporting requirements will include submission of all of the following to your regional amphibian coordinator by no later than September 30.
   - Site Characterization Forms
   - Sample Site & Specimen Photos (digital preferred)
   - Map (refuge brochure map with sampling sites labeled by hand is sufficient)
   - Data Collection Forms (electronic forms preferred)
   - Abnormal Frog Forms
   - Specimen Log (electronic forms preferred)
   - Preserved voucher specimens
   - Documentation of any animals shipped

NOTE: S*ubmitting your data throughout the season as it is collected or at any point before the deadline would be preferred and appreciated!*

National Amphibian Coordinator contact information:

Christina Lydick
US Fish & Wildlife Service
4401 N. Fairfax Drive #322
Arlington VA 22203

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SITE ASSESSMENT & MONITORING SOP

A. Objective

1. To determine suitability of wetlands for frog sampling and guidance for site visits.

B. Procedures

1. Suitable frog sampling sites will primarily be small, isolated wetlands. When possible, sites should be selected to represent any impacted areas of the refuge as well as areas free from any known impacts. Contaminants of concern and surrounding land use should be determined for every selected site. Consult with refuge personnel who might be familiar with the variety of refuge habitats and current and historic activities on/off refuge.

2. When first scouting for suitable sites, it is also helpful to drive around the refuge on warm, humid evenings to locate wetlands where adult frogs are chorusing. If distant chorusing cannot be pinpointed after dark, take GPS coordinates (**see GPS SOP**) to facilitate finding wetland during the day. Considering that the peak breeding/chorusing seasons will vary between species throughout the season, it is helpful to periodically repeat these scouting trips for breeding choruses. Even if you feel sufficient sites have initially been selected, additional suitable sites may be added at any point during the season and may provide valuable data for another taxa that may not choose to breed at one of the previously selected sites.

3. A **Data Collection Form** should be filled out *every time* you visit a site for monitoring purposes. This will provide you the information needed to schedule when to revisit a site later this season AND will provide valuable information for anyone conducting the subsequent seasons monitoring efforts. Even when no tadpoles or metamorphs are found on a site visit, fill out as much of the form as possible: date, Site ID, collectors, ambient air and water temperature, presence of egg masses, water depth and general weather conditions, etc.

NOTE: It is very important to maintain detailed field records of your efforts, given the very unpredictable nature of this project. We may get to the end the season without encountering metamorphs at a particular site where ample tadpoles were found. There would not only be a record to justify your efforts, but the information needed to guide future investigations may become evident in your field notes. The lack of successful breeding activity at suitable sites, or impairment of proper development and subsequent metamorphosis are of concern and would otherwise be difficult to see.

4. When visiting a wetland, you should walk the perimeter looking for metamorphs that jump into the water or into marginal vegetation. If metamorphs are present, capture them using the **Capture SOP**. If metamorphs are infrequently encountered or appear to be absent, dip-net the edges of the pond (sweep the net along bottom and bring it all the way into the bank).

5. If any tadpoles are encountered, record species (when possible) and stage of development (Gosner 1960) of at least 10 tadpoles for each species encountered. Any individuals with both

hind and forelimbs emerged are considered metamorphs and should be collected according to **Capture SOP** (note maximum size restrictions).

6. Determine when or if the site should be visited again based on the following factors:
   a) Ephemerality of the wetland. Does the wetland appear that it will hold sufficient water for long enough to allow development of resident anurans?
   b) Productivity of the wetland. Consider amount of submerged aquatic vegetation, insect life, # tadpoles present, and amount of cover available for frogs. Metamorphs are more likely to stay in/near a productive wetland longer after transformation than in an unproductive mud hole, thus your chances of intercepting them before they emigrate from the area are greater in productive areas. (CAUTION: more productive areas may also be more difficult to sample).
   c) Developmental stage of any tadpoles present (see Section 2 of Training Manual for guidance on the expected window of development for each species). Consider the amount of tree cover at each site when predicting developmental rates. Sites with full sun will most likely experience faster developmental rates than those in heavily wooded or otherwise shady areas.
   d) Projected weather. Because anurans may aestivate in mud or under live and dead vegetation, some wetlands may seem devoid of amphibians during very hot, dry weather, however, after a heavy rain storm or during a period of cool weather, the frogs may reappear. Also be aware that extreme changes in environmental conditions can drive development of tadpoles more quickly than expected. Don't base your site visit schedule through the entire season on the developmental rates encountered early in season, during cooler weather.
   e) Sample ease. Keep in mind that dense vegetation; steep banks and deep mud will all seriously hamper your ability to capture frogs.

NOTE: You should seriously consider the feasibility of sampling for each site early in the season, so you have time to select alternate sites before the breeding window is over. At MINIMUM, four sites per refuge (preferably half impacted, half un-impacted sites) should be monitored throughout the season to ENSURE at least the minimum required collections are feasible. Don't put all your eggs in one basket – so to speak. The resident frogs may not like that particular basket!

7. Keep in mind that bufonids (toads) can successfully breed in extremely short-lived pools and develop very quickly. Even when permanent water supplies are used, newly metamorphosed toads may range widely from the water's edge (this distance increases steadily with age and size). Treefrog metamorphs may begin climbing soon after transformation, so be sure to inspect the entire height of vegetation near the edge of the wetland.

NOTE: ON MONITORING TRIPS TO YOUR REFUGE, TRY TO VISIT EVERY SITE WHEN FEASIBLE; DO NOT ASSUME DEVELOPMENTAL RATES ARE SIMILAR BETWEEN NEARBY SITES OR YOU MAY MISS VITAL SAMPLING OPPORTUNITIES!

C. Reference

Gosner, K.L. 1960. A simplified table for the staging of anuran embryos and larvae with notes on identification. Herpetologica 16:183-190.

CAPTURE SOP

A. Objective

1. To capture 50 to 100 recently metamorphosed frogs. These guidelines will get you started, but once you actually encounter animals, you will likely develop your own technique quickly!

B. Procedure

1. The most efficient time to sample metamorphs is typically when ambient temperatures are mild (i.e. dawn or dusk). Most metamorphosed frogs will inhabit the edges of the wetland, preferentially choosing areas adjacent to shallow water. If there is a high degree of vegetative structure in the wetland, the metamorphs may be found in the interior of the wetland as well.

2. The field team should split into pairs or triplets and begin walking along the edge (or through other promising habitat). Because populations of metamorphs usually exhibit clumped distributions, when a productive area is found, the others should converge on that spot to capture as many frogs as possible before moving the teams elsewhere.

3. Metamorphs vary in size depending on species (consult your regional coordinator for information on species in your area). The listed snout-vent lengths (SVLs) should be used as a general guide. Use your best judgment to determine whether the frogs you have in hand all belong in the same cohort. As the season progresses, it will be very important to note SVL's to ensure you are not resampling the same cohort from the same site.

4. The most effective technique of capturing frog metamorphs is using a dip net . The type of net chosen depends on the habitat. If the edges are fairly open and there is not much debris in the water, a deep, cone shaped net seems to work best. In more dense wetlands, a heavy duty, fairly shallow net should be used. Depending on the characteristics of the site or target species, techniques will vary. Note: there are other acceptable methods for capturing frogs (e.g., sweep nets, traps, electroshocking,e tc.), however, please consult with your regional coordinator prior to using alternative methods. The capture method will depend on the habitat you are sampling

5. You can place the net in front of the selected frog and attempt to "herd" the individual into the net by stepping quickly towards it. This technique can be refined, by having two or more people strategically place their nets to cut off avenues of escape. If working alone, one can also try using a small aquarium net to "herd" the frogs into the larger net. When collecting treefrogs, it is often easier to pick the frogs from the vegetation by hand. However, if they are clinging to sharp edged or other dense vegetation try placing a net on the opposite side of vegetation and coax them to jump off into your net, in order to avoid injury to yourself & to the delicate froglets.

6. Try sweeping your net quickly through the water where a frog is seen (or where one recently jumped into the water). Often this requires the netter to sort through copious amounts of mud and leaf litter before finding the frog; however, this is the most effective technique when sampling densely vegetated wetlands. If frogs are difficult to collect once they have jumped into the water, often they will return to the margins if they are left undisturbed for some time. Make sure to revisit the areas where large numbers of metamorphs were seen (but may have escaped your net).

Spend time working other areas of the wetland or return to site the following day if ambient temperatures are extreme. Once frogs have entered the water during the heat of the day, they tend to stay there, whereas during milder weather, they tend to return to edges more readily if left undisturbed for a short time.

7. Once a frog is captured, it should be placed into a plastic container with a perforated lid, along with a moist paper towel or wet leaves from site. If the individual is a tadpole or still has a tail (>4mm), then it should be placed in a closed lid container or zip-lock bag along with water from the site. In this case, at least half of the container should remain air to prevent the water from becoming anoxic.

ALTERNATIVELY: plastic minnow bags can be used effectively during field capture. They are easily tucked under your belt to free the hands. Be sure to place no more than a few drops of site water to keep bag moist and blow air into bag to provide protective "pillow". Minnow bags should be used to hold no more than 5-10 animals at a time, depending on size/species and should NOT be filled with very much water. Frogs can drown if held in a large volume of sloshing water with no way to take a breath of air!

NOTE - Pickerel frogs (*Rana palustris*) secrete a substance from their skin, which is toxic to other amphibians (Green and Pauley 1987). This species should always be kept in a container separate from the others.

8. Once 50-100 individuals of a single species have been captured, all individuals should be processed. If at least 50 individuals could not be collected at once, animals should be held in a cool place to ensure that none are resampled. The site can be revisited for up to two more days to fulfill the minimum required sample size. Animals should not be held for more than three days before being released back to capture site. It is preferable to process animals on the day of capture for consistency. Do not wait until you get at least 50 to process them, take advantage of the time between visits to process any animals in hand. If even after repeated visits, the minimum of 50 animals could not be collected from a particular site, the **data for any and all metamorphs collected should be recorded.**

C. References

Green, N.B. and T.K. Pauley. 1987. Amphibians and reptiles in West Virginia. Pittsburgh.

DATA COLLECTION SOP

A. Objective

1. To collect the required data and inspect each metamorph for abnormalities.

B. Procedures

1. Once any metamorphs have been collected, the processing may begin. If processing animals that need to be held between additional collection days, make sure to clearly mark which containers have the processed individuals, so that no individuals are resampled.

2. Metamorphs can be held in small groups in the same containers used to collect them if they have sufficient moisture and air. Place the containers in a cooler, layered with blue ice on the bottom, then with a towel or other barrier between the ice & the frog bags. Transfer frogs into marked containers as they are processed. This method is particularly useful if you are working alone. The metamorphs will attempt escape from coolers or other large containers *every time* the lid is opened, which also presents the opportunity for injury to occur.

NOTE: Pickerel frogs *(Rana palustris*) should not be held in same container with any other species. Avoid having too much moisture with *Bufo* (toad) specimens, they can tolerate much drier conditions and are much easier to handle when they are dry. A full collection of toads (50-100) can easily be held in a deep plastic tub during holding or processing, with minimal risk of escape. Just make sure they stay cool & do not desiccate in an air-conditioned building if held overnight.

3. One person should record all data while others measure and inspect each frog. When possible, assign the same duties to the same crewmembers during each collection to keep data consistent throughout the season. If the crew is larger than three, it is helpful to have someone responsible for getting frogs out of holding and to keep track of which ones have/have not been processed. It is also very helpful for handlers to keep their fingers moist (with the exception of handling toads).

4. Identify species as accurately as possible and assign the appropriate code (see **Species Codes Table** in Section 2 of Training Manual). If there is any question on ID, assign the generic code (ex. BUFO for unknown *Bufo* sp.). This should only be the case where similar species coincide.

5. Measure snout to vent length (SVL= from the tip of nose to the cloaca/vent) using a 10 cm ruler. Place ruler on the ventral (belly) side of the selected frog. If there is a tail present, it should be measured separately by placing the edge of the ruler at the base of the tail. Record all information in the appropriate columns on **Data Collection Form**. All collectors and inspectors names should be noted on data sheets.

6. Determine developmental stage for each frog (see **Gosner Stage Chart** in Section 2 of Training Manual). Target stages are 44-46. Any stages after 42 (i.e. all four legs emerged) qualify as metamorphs, however the later staged animals will have more fully calcified bones and will be more

suitable for radiography. If an abnormal animal is found and is still at some stage between 42 and 44, it can be held in a cooler with site water until tail is more fully resorbed, prior to preservation.

CAUTION: Under no circumstances should late stage tadpoles (i.e. animals without hind and/or forelimbs already emerged) be held until their legs emerge! This would create an artificial environment during a critical developmental phase. Any data obtained in this manner must be excluded from the study.

7. Keep in mind, that if disease is evident or if parasitology is warranted, animals should not be held to maximize tail resorption, they should be shipped ASAP (see **Live Specimen Shipping SOP**).

8. After identifying, measuring and staging the frog, the inspector will examine it for any of the abnormalities listed on the standard **Abnormal Frog Forms** (or any others not listed):

a. Hold frog under front legs, with the hind legs dangling down to look for body symmetry. This may take some gentle coaxing of the animal to relax and allow the legs to hang freely.
b. Examine head and jaws for any abnormalities (missing or misplaced eyes, overbite, underbite, unfused, or shortened jaws). Be sure to look at eyes carefully to note the pupil/iris.
c. Examine the front legs, feet, and toes (look for clubbed or missing feet, extra or missing toes, extra or missing limbs, webbing in unusual locations, etc.).
d. Examine the fore and hind legs, feet, and toes.
E. Examine tail. At this point, pay attention to tail length & Gosner stage together. Not abnormalities of the tail [two categories: (1) kinked or bent tail or (2) cysts, lumps, or growths].
F.

NOTE: To ensure all categories are examined, it is advisable to consult an **Abnormal Frog Form** before inspections, particularly if it has been a while since you last inspected frogs.

9. If an abnormality is found, a standard **Abnormal Frog Form** will be completed for each individual abnormal frog. The individual will be placed in a uniquely labeled container or bag and kept moist with site water. See guidance on the form for assigning an appropriate Abnormal Frog ID.

NOTE: DO NOT MAKE ANY FIELD JUDGEMENTS ON CAUSE! A bloody stump that seems apparent to have been caused by a predator (or possibly by your boot or dip-net) may not have looked that way if you had inspected that particular animal one week later. So, in order to avoid bias in our samples, treat ANY animal that is not 100% normal as "ABNORMAL". Just be sure to note any obvious cause of trauma or injury in the comments section of **Abnormal Frog Form**. We don't want to lose any known information about an abnormality and we also don't want to release any injured animals as there may have been another cause which was not evident in the field (i.e. bloody stump of a leg may turn out to be an animal with a malformed pelvis (evident only through radiography), which made that animal more susceptible to being caught and injured by a predator).

10. Normal frogs should always be held until all animals in a collection are processed.

11. Once the entire collection has been processed, consider the following things to determine the fate of your hard-earned collection of metamorphs before releasing any of them at the collection site:

- Do the number and severity of abnormalities fit the criteria for diagnostic tests? (Consult with your regional coordinator for these criteria early in the season. If you are unsure, hold animals until you can contact your coordinator.)
- If parasitology will be required, determine if you need to retain any normal individuals from the collection to complete a total sample of 10 animals.
- Your Regional Coordinator may require you to preserve one normal representative individual for each collection on a refuge (as part of *this* study) as a voucher specimen. If species ID is uncertain, due to the very young age of the animals, consider keeping a few normal animals alive until they develop sufficiently to confirm ID. Keep in mind, this will require some husbandry and possibly several months or longer to exhibit specific diagnostic characteristics, so if you are not so inclined, just preserve one. Please check with your Regional Coordinator for details.
- If your collection consists of any species other than those in genus: *Rana, Bufo,* or *Hyla*, please preserve two normal individuals for comparative radiographic analysis.

12. Once you are certain of the fate of the collection, release any remaining normal frogs by dispersing them along the areas where they were caught. Do not dump them all in one spot because this may facilitate an unnatural predation event. Also, to reduce the possibility of physiological shock, it is preferable not to release chilled animals during the heat of the day. Either hold them until the next morning, or allow them to warm to ambient temperatures before releasing them.

13. Any abnormal animals should then be preserved or prepared for shipping according to the appropriate SOPs.

C. References

Gosner, K.L. 1960. A simplified table for the staging of anuran embryos and larvae with notes on identification. Herpetologica 16:183-190.

SPECIMEN PRESERVATION SOP

A. Objective

1. To preserve frogs and tadpoles so they may be used effectively as diagnostic or voucher specimens.

A. Procedures

1. Prepare a mixture of tricane methanesulfonate (MS-222) with a concentration of 0.5 g/L. This mixture will last for weeks if it is not exposed to sunlight. Store it in a dark bottle and in a cool dark place.

ALTERNATIVELY: A dilute chloretone solution (~10%), which will also keep for weeks if stored in a cool, dark place OR a benzocaine cream can be used to euthanized specimens.

2. Prepare all specimen tags using the standard naming convention (see **Abnormal Frog Form**). Tags should not be affixed to specimens, rather they should be placed inside each frog's individual shipping container. Tags affixed to frogs with wire can impede radiograph quality. And tags affixed with string can become tangled during shipment.

3. Set up a staging area for photo-documentation. Take time to experiment with different backgrounds and camera settings for best focus, lighting, etc. Macro is usually necessary for very small specimens. Note your optimized settings for future photo sessions.

4. Select a container appropriate for the size of specimens you have. Fill with anesthetic, and place one subject in the solution at a time. Cover the jar and allow it to sit until the animal is fully anesthetized (euthanized). Be sure to maintain the identity of each specimen, by keeping the ID tag with the animal. You may choose to set up extra anesthetic chambers, as some individuals may take longer than others to be anesthetized, depending on how fresh the solution is, or number of specimens it has been used on. Specimens can remain in solution temporarily while you photograph/prepare others.

5. Remove euthanized specimen from solution and position in a prone, flattened position, with all toes clearly separated for photography. Lay the appropriately prepared identification tag next to specimen and place a 10 cm ruler in view for scale. Take any additional photographs that might best portray the particular abnormality, by laying specimen on dorsal side or by changing the angle of photography. Photo filenames should be the Abnormal Frog ID (plus sequential numbers at the end for multiple photos of same individual). Often the body is grossly distorted in the preservation process, so whenever possible, take an additional photo of the animal after preservation and note the photo ID with a "P" before Abnormal Frog ID.

TIPS: Use a solid, light colored background. To avoid glare, drain liquid off of each specimen by blotting it on a paper towel just before photography (but be careful that specimens do not dry out completely).

6. If any specimens are larger than one gram, their chest cavity must be opened with a single incision on the ventral side that runs from the cloaca to the sternum. This allows the preservative to quickly reach and preserve internal organs.
7. 75-80% ethyl alcohol (EtOH) should be used as preservative for metamorphs. Do not use formalin unless instructed by your regional coordinator (e.g., a subset of specimens may be prepared for histology).

8. Place specimen in paraffin filled plastic tray for preservation, in prone, flattened position with all toes clearly separated. Using lab tape and dissecting pins to secure specimen in place and carefully fill container with enough preservative to completely cover specimen. If more than one specimen will be prepared, you can tie the identification tag to one of the pins or secure tags to the lid of container in such a way to maintain the ID of each specimen. Be sure to keep specimens from drying out by covering them with a "puddle" of EtOH until all specimens are positioned in tray. Once you fill the tray completely, it is very difficult to properly position any subsequent animals if they are floating.

ALTERNATIVELY: Use several small trays to accommodate specimens separately, but keep in mind this method will require that you use larger volumes of EtOH. Label each container clearly so you know when to remove specimens.

9. After 48-72 hours, carefully remove specimens from fixing chambers. Place specimens and tags into individually labeled jars filled with fresh 70% EtOH for storage. Specimens should be separated by site and should not exceed a 10:1 EtOH:frog volume ratio. Note: larger metamorphs (e.g., leopard frogs) may need to remain in 80% ethanol for a longer period of time, please check with your Regional Coordinator for instructions.

10. If tadpole collections are required, they should be handled differently than metamorphs. Young tadpole specimens will be very fragile, and should be handled delicately and as minimally as possible. Euthanized tadpoles without legs can be fixed by dropping them in a jar of 10% buffered formalin for 24-48 hours. After which, specimens should be moved to fresh formalin for storage. The old formalin can be reused several times, then poured down the sink with copious amounts of tap water to dilute. Late-stage tadpoles with limbs should be preserved the same as metamorphs, with toes separated, etc., if possible. Prop the long tail up at several points using pins, to keep it from twisting or curving unnaturally during fixation.

11. The following information should be noted in Specimen Log for EVERY preserved specimen:
    a. Dates:
        -Field collection date
        -Date specimens fixed
        -Date specimens moved to storage solution
        -Date specimens shipped
    b. Refuge name
    c. Site ID
    d. Abnormal Frog ID (or note as Normal or Voucher specimens)
    e. Species ID Code
    f. Type and concentration of fixative & final preservative used
    g. Name of curator

12. All specimens should be stored in jars, separated by SITE. All jars should be labeled at least with:
    a. Frog ID
    b. Date stored
    c. Type & concentration of solution in jar
    d. Initials of curator

13. All abnormal animals must be documented thoroughly. You will need one photocopy of the **Abnormal Frog Form** for your records, one copy to send to your regional coordinator. **Original abnormal frog forms and a copy of the Site form should accompany specimens when/if shipped for diagnostics.**

Send preserved frogs for radiography to:

Contact Information: Dr. Mike Lannoo Indiana University School of Medicine - TH Holmstedt Hall, Room 135, ISU Terre Haute, IN, 47809 Phone 812-237-2059 Email: mlannoo@iupui.edu	**NOTE: BE SURE TO COPY YOUR REGIONAL COORDINATOR ON ALL DOCUMENTATION & COMMUNICATIONS FOR TRACKING PURPOSES!! SEND THE ORIGINAL ABNORMAL FROG FORM AND A COPY OF THE SITE ID FORM WITH THE FROGS GOING FOR DIAGNOSTICS!**

LIVE SPECIMEN SHIPPING SOP

NOTE: LIVE FROGS SHOULD NOT BE SENT FOR PARASITOLOGY WITHOUT PREVIOUS DISCUSSION/APPROVAL THROUGH REGIONAL COORDINATOR.

A. Objective

1. To pack live frogs and safely ship them for parasitology.

WHICH REFUGES WILL NEED TO SEND FROGS FOR PARASITOLOGY?

Parasitology studies will vary depending on your region so it is important to check with your regional coordinator. These allotted samples are reserved for previously sampled refuges where elevated abnormality rates have been detected. BUT, everyone should keep your coordinator updated on your sampling efforts & results throughout the season.

ONCE APPROVED, HOW DO I SELECT FROGS TO SEND FOR PARASITOLOGY?

Parasitology samples should consist of a total of 10 frogs from one SITE. A SITE is a discrete pool/pond, so sites within a refuge should not be mixed. The sample should be composed of either ALL abnormals from a full collection (50-100 of a single species/cohort). OR, if fewer than 10 abnormals were found in that collection, fill in the sample with normal animals from the same collection to a total of 10. IF >10 abnormals were found in that collection, select the ones with the most severe problems or select individuals that best represent the different types of the most severe abnormalities found. Also, if possible, collect 2-3 adult frogs to send along with the metamorphs. Due to differences in feeding habits, adults often accumulate a different parasite load than metamorphs, and will help provide a more complete picture of the site. NOTE: Toads and Hyla may be sent for parasitological analysis. Non-native species (e.g., bullfrogs in the West) are also acceptable if native species are tough to come by.

B. Procedures

1. **First, call Pieter Johnson to make sure he will be available to receive a shipment.** If frogs will not arrive before Friday, hold frogs in a cool place over the weekend.

2. Place blue ice on the bottom of cooler and cover with several layers of newspaper. Put folded white (not colored or patterned) paper towels on the bottom of small Tupperware/plastic container and saturate with site water or UNCHLORINATED tap water. Place one frog per container. Put perforated lid on container without squishing the frog that will inevitably try to escape. Label the container with the abnormal/normal frog ID#.

3. Include a pre-labeled histology vial for each frog sent. Dr. Johnson has agreed to archive the gonads from each frog, so we need to provide pre-labeled vials to expedite the process.

4. Clearly label the top of container with masking or lab tape with appropriate Abnormal Frog ID or Normal Frog ID. Place the containers on the bed of newspaper in the cooler. Place blue ice on sides of cooler and pack newspaper around the containers and blue ice. **Make sure the blue ice is NOT in contact with the frog containers as the frogs may freeze.** Place several layers of newspaper on top of the containers and then one more layer of blue ice on the top. Fill in with newspapers as needed so that the cargo is snug.

5. Fill out the Parasitology Shipping Form and include a preaddressed FEDEX form for returning your cooler. Also, include the original abnormal frog forms (after making copies for yourself). Note: Dr. Johnson has requested some additional information that is not currently on the abnormal frog or parasitology shipping forms. Thus, please provide him with the full name of the refuge (not just the refuge code) and the GPS

latitude and longitude information for each collection site (this information is on the Site Collection form). Place all documentation in a large Ziploc bag and tape it to the inside lid of the cooler. Secure the closed cooler by taping around the lid several times. Ship via priority overnight.

Contact Information:

Dr. Pieter Johnson
University of Colorado
Ramaley N122
Campus Box 334
Boulder, CO 80309-0334
Phone: 303-492-5623
Email: pieter.johnson@colorado.edu

NOTE: BE SURE TO COPY YOUR REGIONAL COORDINATOR ON ALL DOCUMENTATION & COMMUNICATIONS FOR TRACKING PURPOSES!! SEND THE ORIGINAL ABNORMAL FROG FORM AND COPY OF THE SITE ID FORM ALONG WITH THE FROGS BEING SHIPPED FOR DIAGNOSTICS!!

PRESERVED SPECIMEN SHIPPING SOP

A. Objective

1. To safely ship preserved specimens for radiography or archival purposes.

NOTE: A minimal volume of EtOH is considered a hazardous material when going through US Postal Service or other mail carrier. There is a GREAT deal of liability involved, both for you personally & for the service, so please follow these specific guidelines closely**.**

Alcohol preserved specimens can be shipped non-restricted as long as it conforms to 49 CFR 173.4 - Small quantity exceptions.

B. Procedures (These are the essentials that cover us on frogs from the above mentioned CFR):

1. Select and prepare enough "primary containment vessels" to accommodate all of specimens to be shipped. These primary vessels must be leak proof containers, such as glass, plastic, or metal jars -- as long as it has a thickness of no less than 0.2mm.

NOTE: Ziplocs and seal-a-meal pouches DO NOT qualify for use as primary containment vessels.

2. Specimens should be placed in individual containers and remain separated by collection site.
3. Preserved specimens should be removed from storage EtOH and carefully wrapped in plain white paper towels. When packing the animal in the jar, keep them flat. You may have to have different sized containers for different species of animal. Use the smallest container possible to minimize movement of the frog within the container to minimize damage to fragile extremities.
4. Carefully place the wrapped specimens inside the primary vessel, and saturate completely in 70% EtOH. Once saturated, drain as much liquid from the vessel as possible by inverting the jar for a few minutes, avoid pressing on wrapped specimens. Saturated paper towels in a tightly sealed jar should be sufficient to avoid desiccation of specimens, but make sure the shipment will get to its destination within a few days and that Dr. Lannoo or Dr. Green knows the shipment is coming and that the specimens will need to have ethanol added to the containers upon arrival.
5. The volume of EtOH inside each primary containment vessel must be less than 30mL. (You can ship as many primary vessels together as you need to, but **each** one is allowed only up to 30mL EtOH).
6. The lid of the primary vessels must be secured by "positive means". This means that each sealed jar must be securely taped all they way around the lid.
7. Select an appropriate "secondary containment vessel" that can accommodate all the primary vessels for shipping. It also must be leak proof. You can use either large plastic quart jars to individually hold smaller primary jars, and then fill them with absorbent paper OR use an ice cooler (not styrofoam) that will be shipped back to you (if properly labeled with return address and a pre-labeled FEDEX slip with your account # is included in your shipment).

NOTE: A properly packed, sturdy ice cooler serving as the secondary containment vessel should qualify as the final outer packaging too.

8. Absorbent material must surround the primary vessel(s) in sufficient quantity to absorb the total volume of liquid. Stuffing shredded paper around the jars works well. Shredded paper covers both the requirements for sufficient absorbency and helps to secure the jars from shifting. A layer of bubble wrap between glass jars is a very good idea to prevent breakage, but it is NOT absorbent, so you must add paper too.

9. Again, your secondary container can be the final outer packaging, **if** it is STURDY enough. Officially, it needs to be able to withstand the following:

   - Drop from a height of 6ft, landing at least once flat on bottom, top, long side, short side and once landing on the corner junction of three intersecting edges.

10. Clearly label the outside of the package with the following:

"THIS PACKAGE CONFORMS TO 49 CFR 173.4"

NOTE: It wouldn't hurt to also label the package with FRAGILE / THIS SIDE UP.

11. Make sure to notify the recipient of when to expect your package, include the original abnormal frog form, and copy your regional coordinator on ALL documentation.

** **Alert the recipient that your specimens are being shipped in saturated paper towels, so they will know that they need to open the package immediately and put specimens back in 70% EtOH to avoid desiccation.**

BIOSAFETY DURING AMPHIBIAN HANDLING AND MORTALITY EVENTS

Adapted from: Speare, R., L. Berger, (School of Public Health and Tropical Medicine, James Cook University, Townsville, Australia, 4811); and H. Hines, (Department of Environment, Moggill, Queensland, Australia). 1998. HOW TO REDUCE THE RISKS OF YOU TRANSMITTING AN INFECTIOUS AGENT BETWEEN FROGS AND BETWEEN SITES

A. Objectives
1) Reduce risk of you becoming exposed to a disease agent.
2) Reduce risk of disease transmission between amphibians within infected sites.
3) Reduce spread of disease to amphibians at new sites (long and short distances).

B. Problem
1) Disease agents, pathogenic to frogs, are present in the environment
   - Disease agents include toxins, bacteria, viruses, parasites, fungi
2) Rates of disease transmission within an amphibian population can be increased.
3) Disease can be transmitted to humans or uninfected amphibians at new locations.
4) Potential for long term impact on amphibian populations such as local declines.

C. Background
1) Microorganism
   a) Durable and can survive in a range of environments.
   b) Present in water and substrate as well as infected frogs.
   c) Number of infectious particles available (inoculating dose) can determine outcome.
      - Low numbers of organisms may result in no disease or mild disease
      - High numbers of organisms may result in rapid onset and severe disease.
2) Procedures available to reduce infectivity of durable microorganisms:
   a) Measures may not kill all particles of agent or prevent exposure
   b) Reduction of the number of particles of the agent may improve outcome
   c) Procedures in any disease control are done on a cost-benefit basis:
      - If preventing transmission is critical (i.e. endangered species), expensive and tedious control procedures may be justified to minimize the risk.

D. Reducing risk of transmission between amphibians within infected sites.
1) Do not handle uninfected amphibians during or following contact with sick or dead animals.
2) Invasive procedures, such as toe clipping, on infected amphibians enhance inoculation of disease agent into toes of uninfected amphibians.

E. Handling procedures that reduce disease transmission when handling infected amphibians:
1) Wash and rinse hands well using a disinfectant soap or rinse with an antiseptic
2) Use disposable vinyl gloves for each animal
3) Capture and handle amphibians using new plastic bags for each new animal
4) Prevent contact between amphibian and handler's skin or clothing
   a) Wear protective clothing, chest waders, plastic apron or coveralls that can be rinsed in contact occurs.
   b) Ensure that used gloves and bags do not come in contact with clean ones

5) If invasive procedures are used, decrease the risk of disease transmission:
   a) Use disposable sterile instruments or sterilized previously used instruments
   b) Seal any open cuts (i.e. toe or PIT tag hole) to decrease risk of infection.
      - Use of a cyanoacrylate compound will seal the wound until it heals naturally
   c) Immerse cleaned instruments in a sterilizing solution or boiling water for recommended time (70% methanol for 30 min, 100% methanol and then flamed, 1% glutaraldehyde for 15 minutes or boiling water for 10 minutes).

F. Do not let amphibians come in contact with the disinfectants

G. Do not discard contaminated or disinfected solutions in the water
   1) Use disinfectants at least 200m away from water

H. Reducing risks of spread to new areas
   1) Always visit uninfected sites (typically ephemeral sites) before sites suspected to be infected (typically permanent wetlands)
   2) Long distance movements of handlers greatly increase the possibility of introducing new agents
   3) Disinfect skin before leaving site after handling amphibians
   4) Change clothes, bag and wash
   5) Rinse off visible mud and debris from boots, nets and equipment
   6) Scrub with soap & water (Biodegradable soaps are available at most local outdoors shops, this is preferable if you will be cleaning equipment in the field. If you have access to hose & sewer drains, a general detergent is fine. DO NOT use IODINE based soaps. Do not use combination disinfectant/antibiotic solutions)
   7) Disinfect boots, nets and equipment using a 5% Clorox bleach solution by soaking for 10-15 minutes. DO NOT RINSE. Allow equipment to air dry before going to next site. If possible, having a second set of boots/nets may be useful if sampling between distant sites
   8) Clean outside and underneath vehicle. If mud or water was introduced into vehicle, then scrub floor and pedals with disinfectant
   9) Do not translocate adult amphibians or their eggs or tadpoles over long distances
   10) Consider not returning captive held amphibians or tadpoles to the wild, particularly if they have been in contact with other captive amphibians or tadpoles
   11) If amphibians of high value (threatened or endangered) are being returned to the wild, insist on an examination by a disease specialist prior to release

NOTE: IF AT ANY TIME, YOUR CREW HAPPENS UPON **DEAD OR DYING** AMPHIBIANS IN THE FIELD, PLEASE COLLECT THE CASUALTIES, PUT THEM ON ICE (DON'T FREEZE THEM SOLID, BUT KEEP THEM WELL CHILLED BY PACKING THEM ON PLENTY OF ICE FOR SHIPPING). SEND THEM TO THE NATIONAL WILDLIFE HEALTH CENTER ASAP (SEE CONTACT INFO BELOW). INVESTIGATION OF DISEASE OUTBREAKS AND DIE-OFFS IN WILDLIFE IS THE PRIMARY MISSION OF THE NWHC. SO, ALTHOUGH THAT IS NOT PART OF OUR SURVEYS, PER SE, IT IS OF GREAT INTEREST TO THEM AND THEY WILL TAKE A LOOK AT THOSE SPECIMENS FREE OF CHARGE. TO AVOID ANY CONFUSION ON CHARGES THAT MAY COME THROUGH, MAKE SURE YOU IDENTIFY THEM CLEARLY AS DISEASE OR DIE-OFF ANIMALS AND NOT AS ANIMALS COMING FROM THE USFWS FROG SURVEYS!

IF ANY **DEAD OR DYING** ANIMALS ARE FOUND AT ONE OF YOUR SITES, BIOSAFETY PROCEDURES SHOULD BE IMPLEMENTED IMMEDIATELY ON ALL EXPOSED FIELD GEAR BEFORE VISITING ANY OF YOUR OTHER SITES.

AT MINIMUM, BIOSAFETY PROCEDURES SHOULD **ALWAYS** BE CONDUCTED UPON RETURN TO YOUR FIELD STATIONS OR AFTER VISITING ANY SITES OFF REFUGE OR WHERE YOU OTHERWISE SUSPECT A PROBLEM.

CONDUCTING BIOSAFETY PROCEDURES BETWEEN SAMPLING SITES WITHIN A REFUGE IS NOT TYPICALLY FEASIBLE AND WILL BE LEFT TO THE DISCRETION OF THE RESEARCHER. IF YOUR SITES ARE ALL WITHIN A 50 MILE RADIUS, THEY LIKELY HAVE SIMILAR PARASITE OR BACTERIA LOADS, BUT **USE YOUR BEST JUDGEMENT**. IF YOU HAVE A PARTICULARLY NASTY SITE, CONSIDER CARRYING A SEPARATE SET OF NETS/BOOTS RESERVED FOR USE ONLY AT THAT SITE.

THESE BIOSAFETY PROCEDURES WERE DEVELOPED ORIGINALLY FOR THE FOLKS IN R5, WHERE A SINGLE CREW CONDUCTS MOST OF THE SAMPLING ACROSS SEVERAL STATES. OVER SEVERAL SEASONS, RESEARCHERS HAVE DOCUMENTED THE PROGRESSIVE SPREAD OF PATHOGENS TO SITES THEY VISITED, WHERE PREVIOUSLY NO SIGNS OF PATHOGENS WERE EVIDENT.

If shipping animals, first contact the NWHC to alert Dr. David Green to expect your shipment and how many/type/condition of specimens they will receive.

NWHC:
PH (608) 270-2400
FAX (608) 270-2415

If you cannot reach them by phone, FAX them a note or send an e-mail to Dr. Green (**david_green@usgs.gov**)

Shipping Address:

National Wildlife Health Center
ATTN: DIAGNOSTIC SPECIMENS – WILDLIFE
6006 Schroeder Road
Madison, WI 53711-6223

PLEASE COPY ANY CORRESPONDENCE WITH NWHC TO YOUR REGIONAL AMPHIBIAN COORDINATOR, SHOULD THERE BE QUESTIONS!

GLOBAL POSITIONING SYSTEM PROCEDURES

A. Objective: To collect required geospatial data in the correct format.

Datum (Projection): Data should be collected in WGS84 format (sometimes written as WGS1984). If your GPS unit doesn't support WGS84, the contingent Datum is NAD83. If you collect NAD83 (or any other datum), the datum must be converted to WGS84 before being entered in to the database.

Units (Coordinate System): Points should be collected in Geographic Decimal Degrees (**hddd.ddddd).** If points are collected in Decimal Minutes (hddd.mm.mmmm) or Degrees Minutes Seconds (hddd mm ss.s) then they must be converted to Decimal Degrees before being entered into the database.

NOTE: It is always best to collect the datum and units in the preferred format since there is a loss of accuracy during the conversion process.

Satellites: Ensure that your unit, at the time of collection, is tracking at least 3 satellites (the minimum number required to take an accurate point). Most GPS units will not take a reading unless it has locked on to at least 3 satellites. However there is an override feature on some of the high end units, so it is always prudent to make sure you are tracking a sufficient number of satellites.

WAAS: If your GPS unit is capable of differential correction, make sure it is enabled. Sometimes there will be a small symbol that looks like an antenna on the display, other times you will see the WAAS symbol. Most small units won't have this option, but if you do, turn it on.